Cholesterol Oxides I. Isolation and Determination of Some Cholesterol Oxidation Products

Cholesterol in purified triolein was subjected to saponification by two procedures employing hot alkali and was found to give rise to oxidation products despite precautions. The isomeric 5,6-epoxycholestanols and 7-hydroxycholesterols were stable under saponification conditions, but 7-ketocholesterol and 6-ketocholestanol were destroyed by hot alkali. The oxidation of cholesterol during saponification was not inhibited by the addition of BHT. The determination of a number of standard cholesterol oxides by direct on-column GC was demonstrated with good resolution. Their order of elution was different from that of the TMS-derivatized products.

The lability of cholesterol to oxidation has been amply documented, and well over 60 products resulting from autoxidation, photooxidation and enzymatic action have been described (1). Several of the cholesterol oxidation products have been implicated in adverse human health effects (2), and current knowledge of their biological and biochemical activities has been summarized (3). Of particular concern have been reports (4–10) that provide evidence that certain cholesterol oxides show angiotoxicity, cytotoxicity, atherogenicity, mutagenicity and carcinogenicity.

Some cholesterol-containing foods are subject to oxidizing conditions during various stages of processing or preparation. Conditions such as exposure to elevated temperatures in contact with air for even a brief period, or prolonged storage in air at ambient temperatures, are likely to lead to the formation of cholesterol oxides. Such oxides, when formed, are present in foods in rather low concentration, and their isolation and determination have presented challenging analytical problems. Consequently, relatively few food products have been examined for sterol oxide content in the past (1,11). Recently, however, development of appropriate analytical methods has led to the demonstration of cholesterol oxides in eggs and egg products (12–17), cheeses (18), tallow (19), meats (20) and other foods, although quantitation has been complicated by artifact formation.

Because of the low concentration of cholesterol oxides in foods, most attempts to measure these products require isolation and concentration steps to precede the actual determination. Previous workers in the field frequently have employed saponification of the extracted lipids with hot alkali as an important part of the enrichment procedure (15,17–19,25,26). This step frees the bulk of the lipids from the sterol residue and also converts esterified cholesterol to the free sterol. Nevertheless, some important cholesterol oxides, notably 7-ketocholesterol, have long been known to be unstable to hot, aqueous alkali (11,27). Smith (1) has reported the artifactual formation of cholesterol oxidation products during the course of the saponification procedure.

Cholesterol oxidation product mixtures of varying complexities have been analyzed frequently by HPLC (1,15,17,18,30–34) or by GC of their silylation products (1,16,35–37). Some work on the direct GC injection of underivatized cholesterol oxides also has been reported (12,19,37–39). In spite of very significant advances during the past 5–6 yr, HPLC techniques apparently have not yet reached the point where they are useful in the separation and quantification of complex mixtures of cholesterol oxides. Application of GC methods has been somewhat more successful despite problems of incomplete derivatization and peak resolution. Capillary GC, especially in combination with direct on-column injection, has increased the capability of GC as a powerful tool in the analysis of cholesterol oxides. In this regard the studies of Missler et al. (16) and Krull et al. (37) are particularly noteworthy.

In contrast to prior investigations, the current work was undertaken to study the effect of saponification on several cholesterol oxidation products, to investigate the direct GC determination of complex, underivatized cholesterol oxide mixtures, and to study artifact formation during analysis. We have followed the lead of Missler et al. (16) in the use of semipreparative HPLC to separate the cholesterol oxidation products from the bulk of cholesterol and other lipids, and in the GC analysis of the enriched oxide fraction.

EXPERIMENTAL PROCEDURES

Materials and reagents. Cholesterol oxide standards were purchased from Sigma Chemical Company (St. Louis, Missouri), Steraloids, Inc. (Wilton, New Hampshire) or Research Plus, Inc. (Bayonne, New Jersey). Cholesterol, ash-free, purchased from Sigma Chemical Company, was purified by the dibromide method (21) and further refined by HPLC. Cholesterol-5β,6β-epoxide was prepared from cholesterol via cholesterol-3β,5α,6β-triol (22) and the corresponding triacetate (23) by the method of Chicoye et al. (24).

Triolein, practical grade, purchased from Sigma Chemical Company, contained unsaponifiable matter that interfered with the GC determination of cholesterol oxides. To remove these interfering contaminants, 520 g of oil was treated with 150 g of silicic acid and 30 g of anhydrous, powdered calcium chloride with stirring under nitrogen at 60–70 C for 2 hr. Activated carbon (5 g) was added and the mixture filtered and stored under nitrogen.

All solvents used were "distilled in glass grade" and were degassed by vacuum filtration through a 0.2 μm filter. Water was double deionized, glass distilled and filtered via a Norganic (Millipore) filter. TLC plates, silica gel G and GHL (250 microns), were purchased from Analtech (Newark, Delaware).

PROCEDURES

Saponification. Purified cholesterol was added to purified triolein with gentle heating (40 C) and stirring to prepare a homogeneous solution containing 10 mg cholesterol per gram of solution. This solution was used in subsequent saponification studies. The AOAC saponification method (29) was used without modification. The dry column saponification method (28) was applied as described, except that the following minor modifications were made: The sample size was reduced to 2.5 g (from 5.0 g), the mortar size was reduced to 275 ml (from 400 ml), the oven temperature was 110–120 C, and a nitrogen atmosphere was maintained over the sample during heating and cooling. Prior to HPLC the solvent (dichloromethane) was removed from the sample under a stream of nitrogen. The sample was dissolved in 1 ml ethyl acetate, the solution filtered through a 0.45 μm filter (4 mm Millex filter, Millipore) and the filter and flask washed with 4 × 1 ml ethyl acetate.

Liquid chromatography. Semipreparative HPLC was performed using a Waters Associates HPLC system consisting of a model 721 system controller, Model 730 data module, model R-401 differential refractometer, two model 6000A pumps, and a model U6K injector with a 3 ml sample loop. The normal phase separation was performed on a 7.8 mm × 30 cm μ-Porasil column. Cyclohexane/ethyl acetate (1/1) was the mobile phase with initial flow of 1 ml/min and a step increase to 5 ml/min at 30 min. The run was terminated at 60 min and the column washed with 50 ml acetonitrile before being returned to initial conditions. A standard solution of purified cholesterol, cholestanol-5α,6α-epoxide and cholestane-3β,5α,6β-triol was injected at the beginning of each day to determine oxide fraction collection times.

Filtered samples in ethyl acetate were blown dry under nitrogen at 40 C. 6-Ketocholestanol (10μg), used as internal standard, was added to the dry sample, followed by 500 μl of ethyl acetate/cyclohexane (1/1). The solution was loaded into the sample loop. Flask and syringe were rinsed by an additional 2 × 500 μl of the mobile phase, the rinse was also loaded into the sample loop, and the sample solution was injected. The oxide fraction was collected starting midway between cholesterol and the 5α-epoxide peak and ending after the trailing peak of the triol. The collection flask was kept under helium atmosphere, protected from light and cooled in a dry ice/acetone bath. The collected oxides were stripped of solvent under nitrogen on a rotoevaporator, and transferred with dichloromethane (3 × 1 ml) to a 1-ml volumetric flask from which the solvent was removed during the transfer under a stream of nitrogen. Finally, the sample was reconstituted to 1 ml with ethyl acetate.

Gas chromatography. Gas chromatography was performed on a Perkin-Elmer Sigma 2000 capillary GC equipped with a flame ionization detector and a cooled on-column capillary injector. Data were collected and integrated on a Perkin-Elmer LCI-100 computing integrator. The column was a 0.2 mm ID × 25 m bonded phase 5% phenylsilicone column with 0.33 μm film thickness (Hewlett-Packard Ultra #2). A 0.32 mm I.D. × 2 m deactivated uncoated fused silica retention gap preceded the column by means of a butt connector (Valco). Helium was the carrier gas at 36 cm/sec. Initial column temperature of 100 C was held 5 min, then programmed 30 C/min to 260 C, then 0.5 C/min to 300 C. Detector temperature was 325 C and the injector was air cooled.

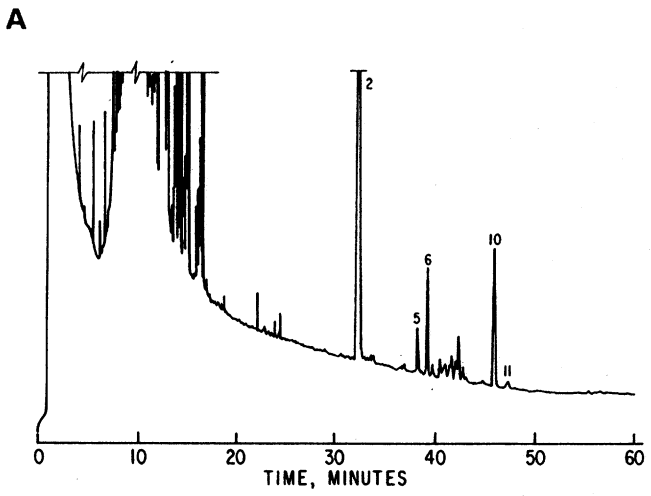
As a measure of the sensitivity of the direct GC procedure, the minimum detectable limit (MDL) was determined for a number of standards. At a signal to noise ratio of 2:1 the following MDL values were obtained: cholesterol, 325 pg; cholestanol-5α,6α-epoxide, 293 pg; 6-ketocholestanol, 265 pg; cholestanol-3β,5α,6β-triol, 498 pg.

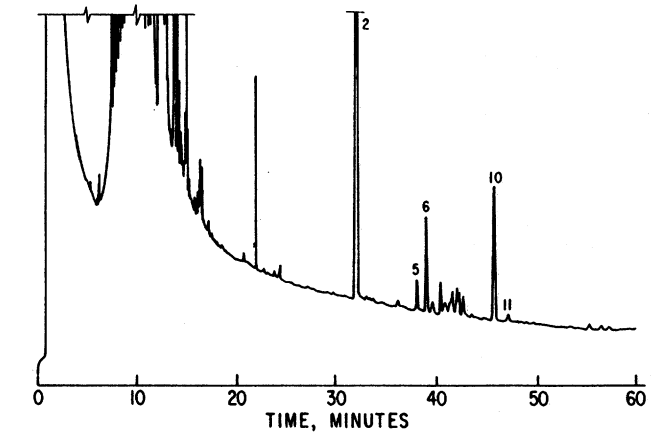
Silylation. Samples to be silylated were transferred to a 1-ml Reacti-Vial (Pierce) and blown dry with nitrogen. Dimethylformamide (0.5 ml) and BSTFA (Pierce) (0.5 ml) were added and the solution heated at 75 C for 1 hr. The sample was concentrated to 100 μl with a stream of nitrogen, and 1 to 2 μl of the resulting solution was injected on-column into the GC.

Thin layer chromatography. Prior to use, plates were washed by development with chloroform:methanol (2:1, v/v) and activated overnight in an air oven at 115 C. Silica gel GHL plates were used for analysis. Spotted samples were developed with heptane:ethyl acetate (1:1, v/v); the plates were dipped in 10% aqueous copper sulfate solution containing 8.5% phosphoric acid (w/v), drained and charred. Silica gel G plates were used for sample isolation. Streaked samples were developed with heptane:ethyl acetate (1:1, v/v), and the two edges of the plate (2.5 cm) were snapped off, dipped into copper sulfate solution and charred to locate bands. The latter were scraped from the main part of the plate onto weighing paper and transferred to a 10-cc syringe fitted with a 0.45-μm Millipore filter. Elution with acetone into a tared weighing container was followed by solvent removal and weighing of the residue.

RESULTS AND DISCUSSION

Autoxidation of cholesterol during saponification was studied with the use of a model system consisting of a mixture of purified cholesterol and triolein (10 mg/g mixture). Both components of the solution were free of peaks eluting (GC) in the region of cholesterol oxides. Peanut oil and imported olive oil, both purchased at a local market, also were tried as base oils, but both contained significant amounts of impurities that eluted from the GC in the cholesterol oxides region and could not be readily removed by prior purification. Two methods of saponification were used, a dry column procedure (28) and the official AOAC procedure (29). The unsaponifiable materials obtained by either method were further enriched in cholesterol oxides by semipreparative HPLC, and the eluate containing the oxides was concentrated and then analyzed by GC. For comparison, an aliquot of the solution of purified cholesterol in triolein was subjected to HPLC enrichment without prior saponification, and after concentration, the oxides fraction was subjected to GC analysis. Results are shown in Figure 1 and corresponding peak assignments are indicated in Table 1. It should be noted that samples subjected to saponification (2.5 g oil containing 25 mg cholesterol) were larger than those that were not saponified (0.25 g oil containing 0.25 mg cholesterol) but injected directly into the HPLC. This was necessitated by the capacity limit of the HPLC column. The same amount (10 μg) of the internal standard, 6-ketocholestanol, was added to all samples prior to HPLC enrichment. Figure 1 shows that, even without the





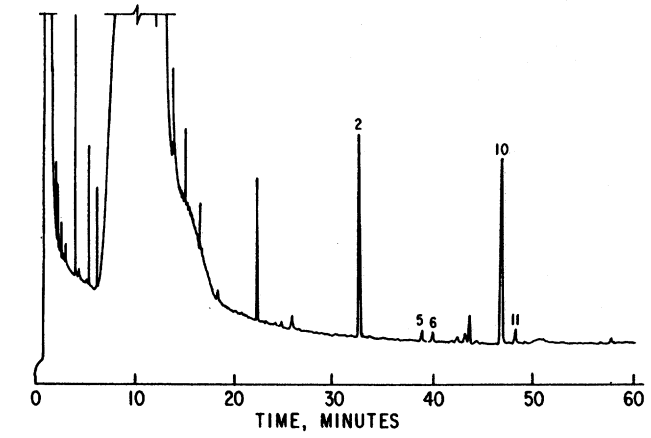


FIG. 1. Effect of saponification on oxide formation, direct GC of oxides. (A) Dry column saponification of 2.5 g oil; (B) AOAC saponification of 2.5 g oil; (C) no saponification, oxides in 0.25 g oil. Peaks numbered as in Table 1. For oil composition, saponification conditions and GC details, see experimental section.

TABLE 1

Direct GC of Standard Cholesterol Oxides

Peak no.	Compound	Kovats index
1	Tetradecyl tetradecanoate (ref.)	—
2	Cholesterol	3123
3	3-Ketocholestanol	3168
4	3,5-Cholestadien-7-one	3208
5	5β,6β-Epoxycholestanol	3252
6	5α,6α-Epoxycholestanol	3271
7	7α-Hydroxycholesterol	3320
8	25-Hydroxycholesterol	3327
9	7β-Hydroxycholesterol	3330
10	6-Ketocholestanol	3394
11	7-Ketocholesterol	3417
12	5-Hydroxy-6-ketocholestanol	3412
13	5α,6β-Dihydroxycholestanol	3537

saponification step, compounds eluting in the cholesterol oxides region and presumed to be oxidation products were generated. Further exploration of this point is described below. It is also apparent that the saponification step contributes to the generation of additional oxidation products, regardless of the method of saponification. From GC retention time data it appears that some of these additional products are isomeric 7-hydroxycholesterols.

The saponified samples contain much less 7-ketocholesterol (0.05 ± 0.02 μg/mg [mean ±1 S.D.] cholesterol) than those that were not saponified (0.49 ± 0.03μg/mg cholesterol). The 7-keto derivative is usually a prominent product of cholesterol autoxidation in solution or dispersion. Other authors (1,11,24) have reported the instability of 7-ketocholesterol toward hot aqueous alkali, and they have reported that one of the degradation products is 3,5-cholestadien-7-one. We subjected 7-ketocholesterol in purified triolein to dry column saponification and found that the compound is almost completely degraded to less polar (by TLC) products including 3,5-cholestadien-7-one. The latter elutes before cholesterol in our HPLC enrichment procedure and therefore is excluded from our enriched cholesterol oxides fraction. Our internal standard, 6-ketocholestanol, also was unstable under saponification conditions, although its degradation was slower. After the normal dry column saponification period of 20 min, only 58% of 6-ketocholestanol could be recovered by preparative TLC, while after one hr only 20% was recoverable. Therefore, this internal standard was not added until after the saponification step. Cholestanol-5α,6α-epoxide in purified triolein was saponified by the dry column procedure. After one hr at 114–122 C, no degradation of the α-epoxide was detectable by TLC. Similarly, cholestanol-5β,6β-epoxide, 7α-hydroxy- and 7β-hydroxycholesterol appeared to be stable to one-hr dry column saponification. Previous reports (11,12) that the isomeric epoxides are unstable to hot alkali could have been due to incomplete recovery.

Dry column saponification of purified cholesterol in triolein was carried out in the presence of 0.3% BHT as antioxidant in an attempt to reduce autoxidation of cholesterol in this procedure. No substantial reduction in the formation of autoxidation products was observed.

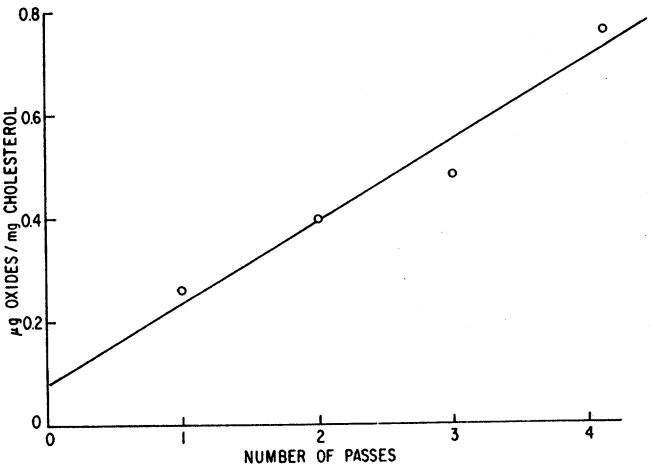


FIG. 2. Oxides formed during HPLC and GC analysis (see discussion section).

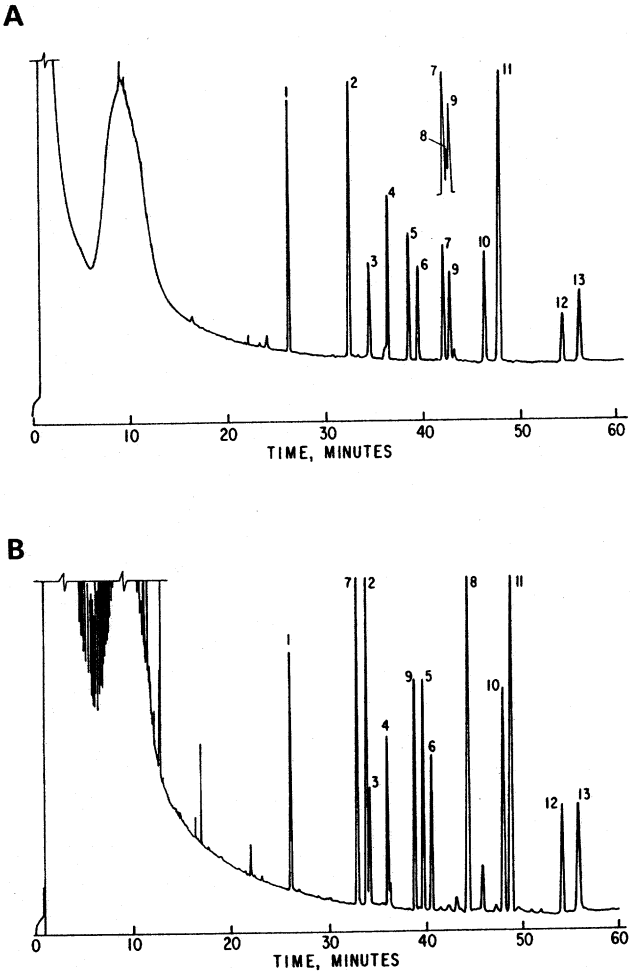


FIG. 3. GC of standard cholesterol oxides. (A) underivatized oxides, the insert shows 25-hydroxycholesterol only partly resolved from the isomeric 7-hydroxycholesterols; (B) silylated oxides. Peaks numbered as in Table 1.

Because BHT as well as other common phenolic antioxidants elute before cholesterol in our HPLC enrichment procedure, they are unable to prevent artifact formation during subsequent steps.

From Figure 1 it is apparent that even without saponification some cholesterol oxidation products are formed during the HPLC/GC procedure. Such compounds are not found either in the purified cholesterol examined directly by GC, or in the unsaponifiable residue of the purified triolein. In spite of the usual precautions taken, i.e. exclusion of air, nitrogen blanketing, low temperatures and exclusion of light where possible, autoxidation of cholesterol during analysis can be minimized but not completely eliminated. This observation has also been made by Missler et al. (16). To obtain a measure of the oxidation products formed during the combined HPLC/GC procedure, purified cholesterol was injected into the HPLC, both the cholesterol and the oxides fractions were collected, and only the cholesterol fraction was recycled three times. The fourth oxides fraction was then analyzed by GC, the third fraction added to the fourth and the mixture analyzed, and so on. The results are plotted in Figure 2. The slope of the line indicates that in our hands 0.16 μg of oxides are formed artifactually per milligram of cholesterol per pass.

The analysis of broad mixtures of standard cholesterol oxides by GC without prior derivatization has not been reported, although GC separations of underivatized isomeric oxide pairs, e.g. the 5,6-epoxides (12,39) and the 7-hydroxychlolesterols (38), have been described. We have analyzed a mixture of 11 standard cholesterol oxides (Table 1) by on-column, capillary GC without and with prior silylation. Results are shown in Figure 3. The underivatized oxides separated quite well except in the area of the isomeric 7-hydroxycholesterols where in the presence of 25-hydroxycholesterol a cluster of poorly resolved peaks appeared (inset, Fig. 3a). Silylation of the oxides changed their order of GC elution (Fig. 3b) and permitted the separate measurement of the isomeric 7-hydroxycholesterols as well as the 25-hydroxycholesterol. Isomeric 5,6-epoxycholestanols, free sterols, as well as trimethylsilyl ethers, gave good baseline separation.

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